The synergistic effects of plant-derived phytonutrients on recombinant antigen vaccination against avian coccidiosis

Sung Hyen Lee[a], Hyun S. Lillehoj[a], Seung I. Jang[a], Kyung Woo Lee[a], Myeong Seon Park[a], Duk Kyung Kim[a], Misun Jeong[a], David Bravo[b]

[a]Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, ARS-USDA, Beltsville, MD, USA
[b]Pancosma S.A., Geneva, Switzerland

INTRODUCTION

- **Chicken**
  - our major protein source & economically important food animal chicken (105 Lb/person/y, 25%), Beef (66 Lb, 47% of meat expenditure), pork (49 Lb, 25%)…
  - well-characterized innate immune systems (Lillehoj et al, 2005,…2008..; Lee et al., 2005,…2010, 2011)
- **Coccidiosis**
  - an intestinal disease caused by multiple species of *Eimeria*
  - an economically important disease for the poultry industry worldwide
  - due to the emergence of drug-resistant parasites, developing drug-free disease control strategies are needed
  - Eimeria recombinant protein vaccines offer less efficacious than coccidiostats and live vaccines.
- **Phytonutrients/plant extracts**
  - enhanced *in vitro* parameters of immunity (lymphocytes proliferation, Nitrogen oxide (NO) production, tumor cell or parasite cytotoxicity, cytokine level, etc.)
  - reduced *in vivo* infection against avian coccidiosis (body-weight, gut lesions, oocyst output, serum Ab response)
  - **Carvacrol (V):** anti-bacteria (strains e.g. E. coli and Bacillus cereus)
  - **Cinnamaldehyde (A):** fungicidal, antimicrobial, anti-cancer activity.
  - **Capsicum oleoresin (C):** anti-fungal, anti-microbial, anti-cancer effects.

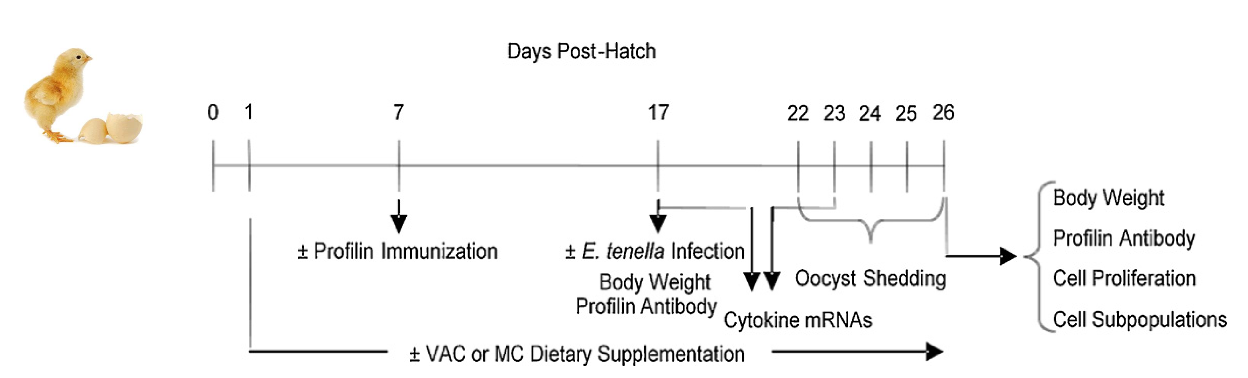
Table 1. Three most reliable gene networks associated with each phytonutrient

Treatment	Network No.	Top Functions*	Focus Genes	Score
Carvacrol	1	Cell Morphology, Cellular Assembly and Organization, Cellular Function and Maintenance	14	29
	2	Cell-To-Cell Signaling and Interaction, Tissue Development, Cellular Movement	12	24
	3	Gene Expression, Endocrine System Development and Function	12	24
Cinnamaldehyde	1	Antigen Presentation, Humoral Immune Response, Inflammation	18	39
	2	Cardiovascular System Development and Function, Tissue Morphology, Drug Metabolism	14	28
	3	Gene Expression, Cellular Development	14	27
Capsicum oleoresin	1	Gene Expression, Cardiovascular System Development and Function, Cellular Growth and Proliferation	23	39
	2	Developmental or Genetic Disorder, Neurological system	19	28
	3	Carbohydrate Metabolism, Cardiovascular System Development and Function, Hepatic System	15	23

OBJECTIVE

- **To investigate hypothesis that the phytonutrients mixture, VAC, would enhance immunity against coccidiosis in broiler chickens.**
- **To evaluate the synergistic effects of VAC on recombinant Ag vaccination against avian coccidiosis.**

MATERIALS AND METHODS



1. Experimental animals and diets

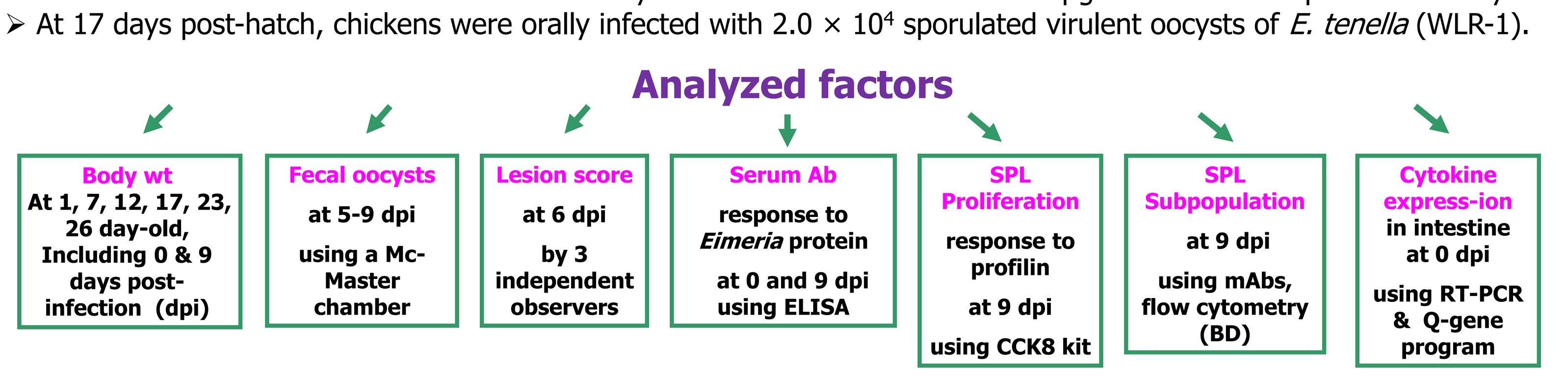
- One-day-old broiler chickens (Ross/Ross) were housed in Petersime brooder units, in 4 groups (12 chickens/group).

Groups	CON	CON	CON-V	VAC-V
E. tenella	-	+	+	+
Vaccine (V)	-	-	+	+
VAC mixture	-	-	-	+

- The birds were kept in brooder pens for 2 weeks and transferred to hanging cages (2 birds/cage) post infection.
- Diet: **standard** diet or the standard diet supplemented with **VAC** for 26 days from 1 day old.
  VAC: 5 mg/kg **carvacrol** (**V**), 3 mg/kg **cinnamaldehyde** (**A**), 2 mg/kg **capsicum oleoresin** (**C**)

2. Profilin immunization and experimental infection

- Recombinant profilin was expressed in E. coli and purified.
- Chickens were mock immunized subcutaneously with PBS or vaccinated with 50 µg of recombinant profilin at 7 day-old.
- At 17 days post-hatch, chickens were orally infected with 2.0×10^4 sporulated virulent oocysts of *E. tenella* (WLR-1).



3. Body weight and fecal oocyst shedding

- Body weights were measured at 1, 7, 12, 17, 23, 26 day-old including 0 and 9 days post-infection (DPI).
- Fecal oocysts were collected daily between 22 and 26 days post-hatch (DPI 5 and 9).
- Oocyst numbers were determined using a McMaster chamber according to the formula:
  total oocysts/bird = (oocyst count × dilution factor × [fecal sample volume/counting chamber volume])/2.

4. Profilin serum antibody levels

- Blood samples (4 chickens/group) were collected by cardiac puncture following euthanasia at DPI 0 and 9.
- Sera were used in an ELISA to measure profilin-specific antibody responses.
- 96-well microtiter plates were coated overnight with 1.0 µg/well of purified recombinant profilin.
- Diluted sera (1:50) were added (100 µl/well), incubated with agitation for 2 h at room temperature.
- Bound antibodies were detected with peroxidase-conjugated rabbit anti-chicken IgG and 3,3,5,5-tetramethylbenzidine substrate.
- Optical density at 450 nm was measured with an automated microplate reader (Bio-Rad, Richmond, CA).

5. Spleen lymphocyte proliferation

- At 26 days post-hatch (DPI 9), spleens (4/group) were removed.
- Cell suspensions were prepared by gently flushing through a cell strainer.
- Lymphocytes purified by density gradient centrifugation were adjusted to 5.0×10^6 cells/ml in RPMI medium containing 10% fetal bovine serum, and 100 g/ml streptomycin and incubated with medium alone or with 20 µg/ml of profilin in 96-well plates in a humidified incubator at 41 ◦C and 5% CO2 for 24 h.
- Cell proliferation was measured using WST-8 at OD450 using a microplate spectrophotometer (Bio-Rad).
- Lymphoproliferation was expressed as stimulation index equal to the mean OD value of the profilin-stimulated group divided by the mean OD value of the medium-only stimulated group.

6. Intestinal cytokine mRNA levels

- Cecal tonsils were obtained from non-infected and infected chickens at 17 days post-hatch (DPI 0) (4/group).
- The mucosal layer was carefully scraped away using a surgical scalpel and total RNA was extracted using TRIzol.
- RNA was reverse-transcribed using the StrataScript first-strand synthesis system (Stratagene, La Jolla, CA).
- Amplification and detection were carried out using equivalent amounts of total RNA using the Mx3000P system and Brilliant SYBR Green qPCR master mix (Stratagene).

Table 1. Oligonucleotide primers used for quantitative RT-PCR of chicken cytokines.

RNA target	Primer sequences	PCR product size (bp)	Accession No.
GAPDH	F: 5′-GGTGGTGCTAAGCGTGTTAT-3′ R: 5′-ACCTCTGTCATCTCTCCACA-3′	264	K01458
IFN-γ	F: 5′-AGCTGACGGTGGACCTATTATT-3′ R: 5′-GGCTTTGCGCTGGATTC-3′	259	Y07922
IL-6	F: 5′-CAAGGTGACGGAGGAGGAC-3′ R: 5′-TGGCGAGGAGGGATTTCT-3′	254	AJ309540
IL-17F	F: 5′-CTCCGATCCCTTATTCTCCTC-3′ R: 5′-AAGCGGTTGTGGTCCTCAT-3′	292	AJ493595
TNFSF15	F: 5′-CCTGAGTATTCCAGCAACGCA-3′ R: 5′-ATCCACCAGCTTGATGTCACTAAC-3′	292	NM010245578

7. Lymphocyte subpopulation

- At 26 days post-hatch (DPI 9) chickens (4/group).
- Single cell suspensions of peripheral blood lymphocytes (PBL) : 1.0×10^7 cells/ml in FCA buffer.
- The cells were incubated with mouse monoclonal antibodies (mAbs) specific for chicken major histocompatibility complex (MHC) class II, CD4, CD8, K1, T cell receptor 1 (TCR1), or TCR2 surface proteins.
- The cells were incubated with fluorescein isothiocyanate-labeled goat anti-mouse IgG secondary antibody.
- The cells were washed three times with FCA buffer, and fluorescence was then analyzed with 1.0×10^4 viable cells using a FACSCalibur (BD, Boston, MA).

8. Statistical analyses

- Statistical analyses were performed using SPSS software (SPSS 15.0 for Windows, Chicago, IL).
- All data were expressed as the mean ± SEM values.
- Comparisons of the mean values were performed by one-way analysis of variance, followed by the Duncan's multiple range test, and differences were considered statistically significant at $P < 0.05$.
- Values not sharing the same letter are significantly different according to the Duncan's multiple range test.

RESULTS

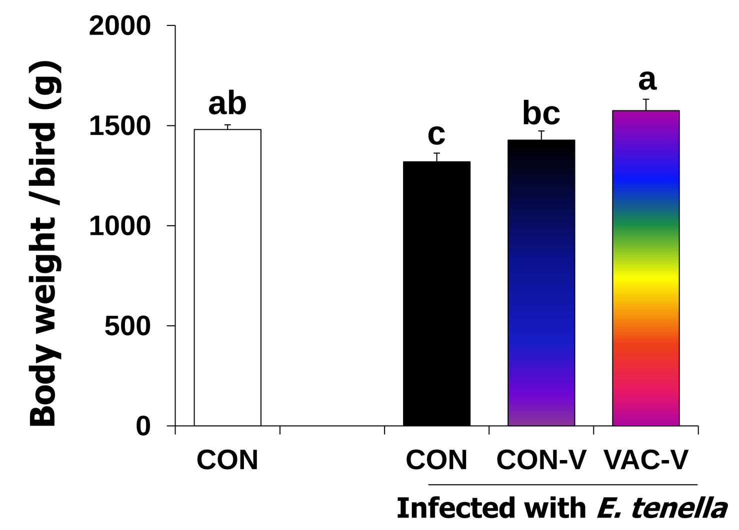


Fig. 1 Effect of dietary supplementation with VAC on body weight at DPI 9.

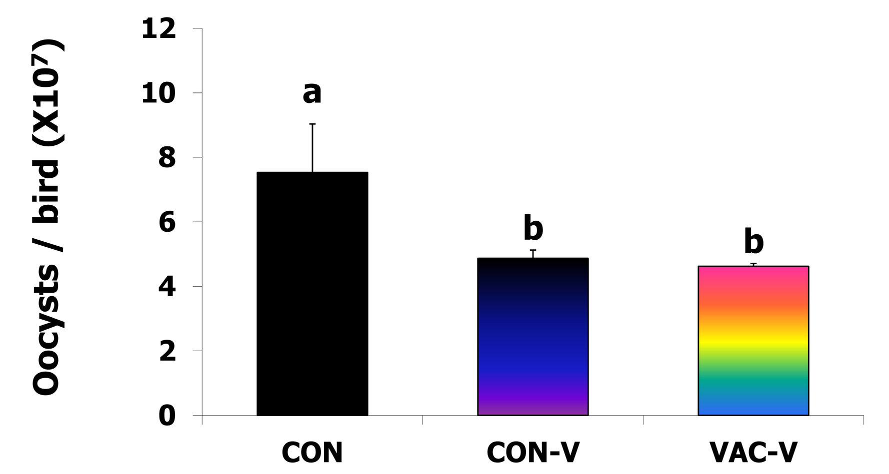


Fig. 2. Effect of dietary supplementation with VAC on fecal oocyst shedding between DPI 5 and 9.

[a, b, c] Values not sharing the same letter are significantly different according to the Duncan's multiple range test.

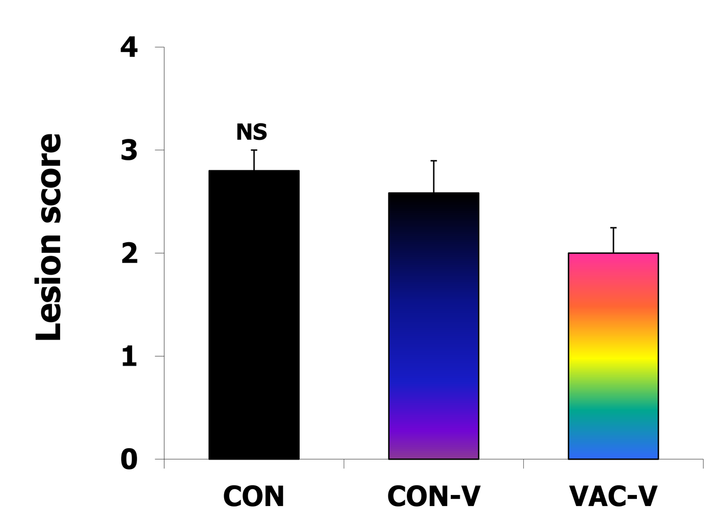


Fig. 3. Effect of dietary supplementation with VAC on lesion score at DPI 6.

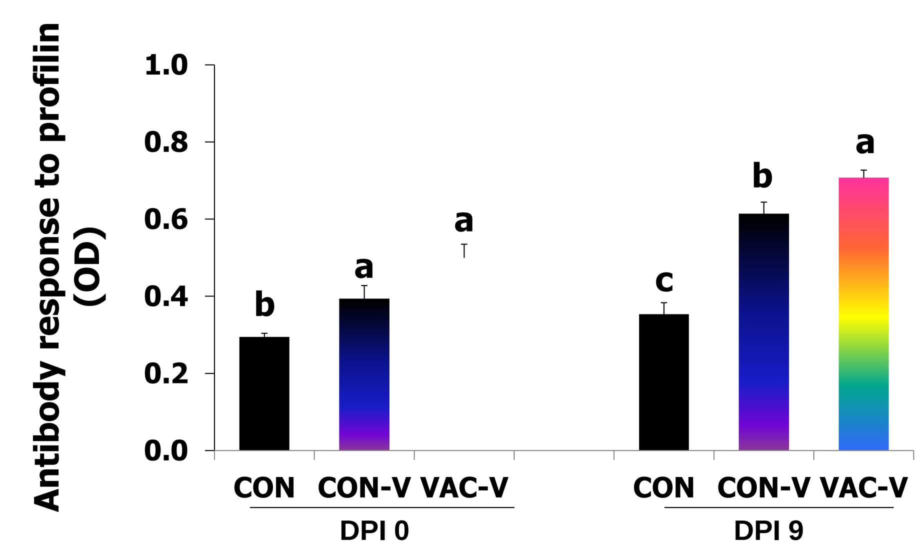


Fig. 4. Effect of dietary supplementation with VAC on profilin serum antibody levels.

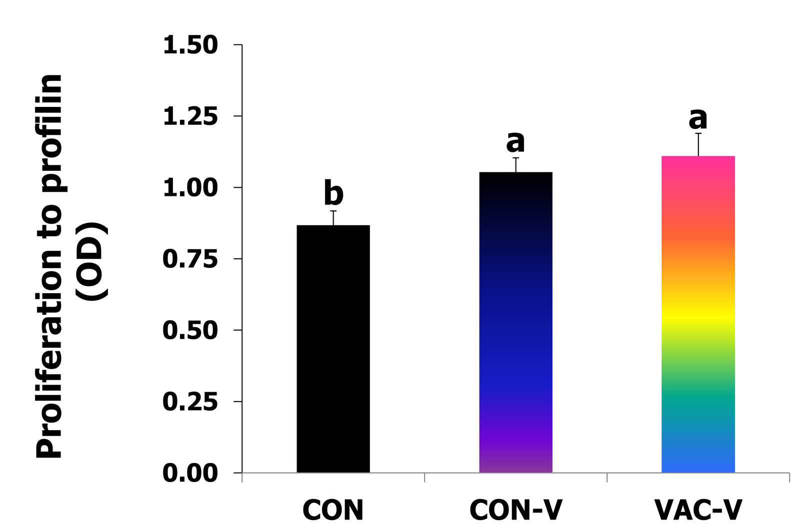


Fig. 5 Effect of dietary supplementation with VAC on spleen lymphocyte proliferation at 26 day-old (DPI 9).

Table 2. Effect of dietary supplementation with VAC on PBL subpopulations.

mAb/%	CON	CON-V	VAC-V
MHC2	10.6 ± 1.3[b]	21.3 ± 1.8[a]	18.6 ± 1.1[a]
CD4	7.9 ± 1.0[b]	17.3 ± 1.6[a]	16.2 ± 1.1[a]
CD8	11.4 ± 1.4[b]	23.3 ± 1.8[a]	23.3 ± 1.9[a]
K1	1.9 ± 0.2[b]	2.1 ± 0.1[b]	3.9 ± 0.2[a]
TCR1	2.1 ± 0.3[NS]	3.6 ± 0.3	2.8 ± 0.1
TCR2	6.9 ± 0.6[NS]	9.1 ± 0.5	13.4 ± 0.8

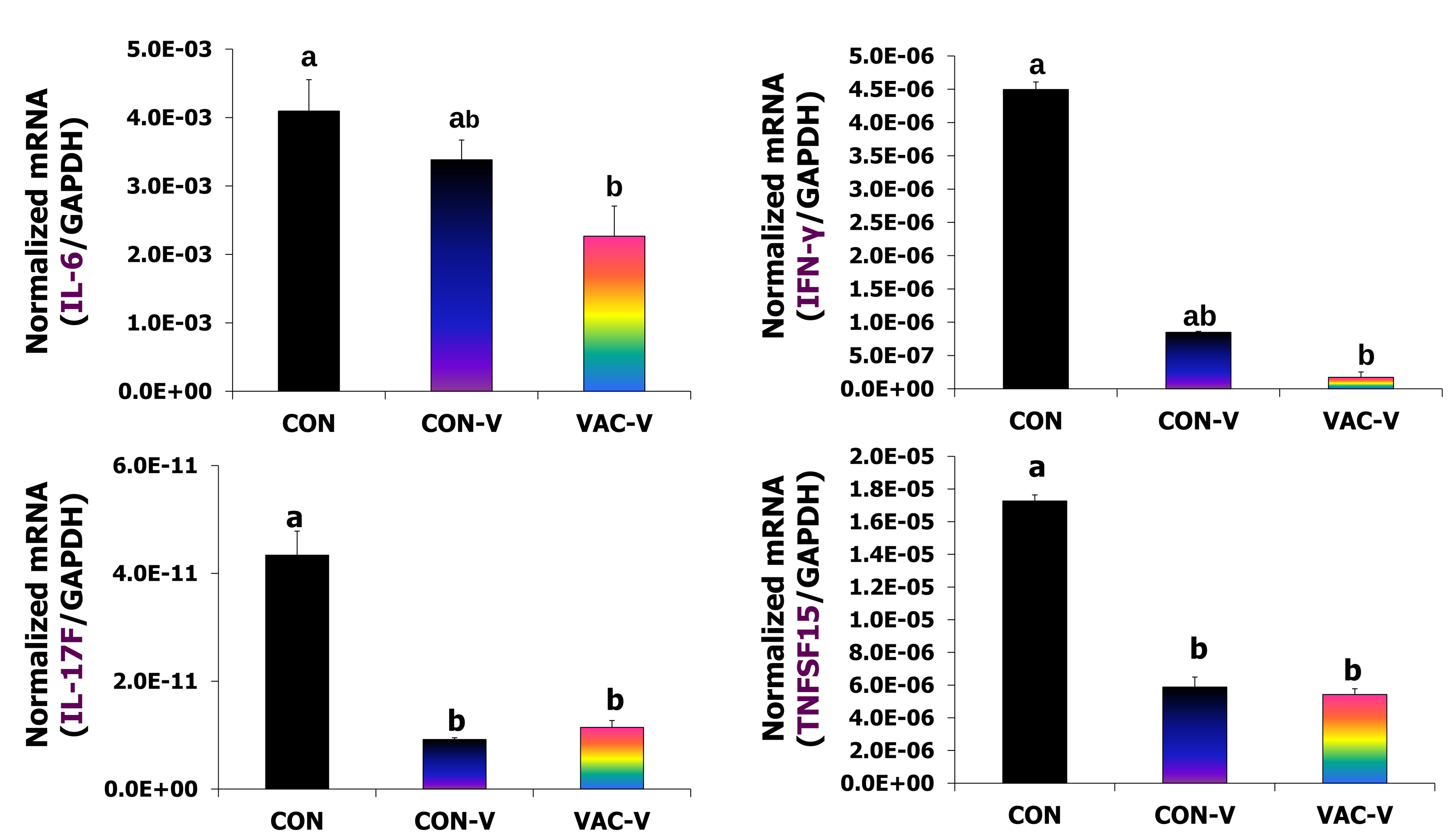
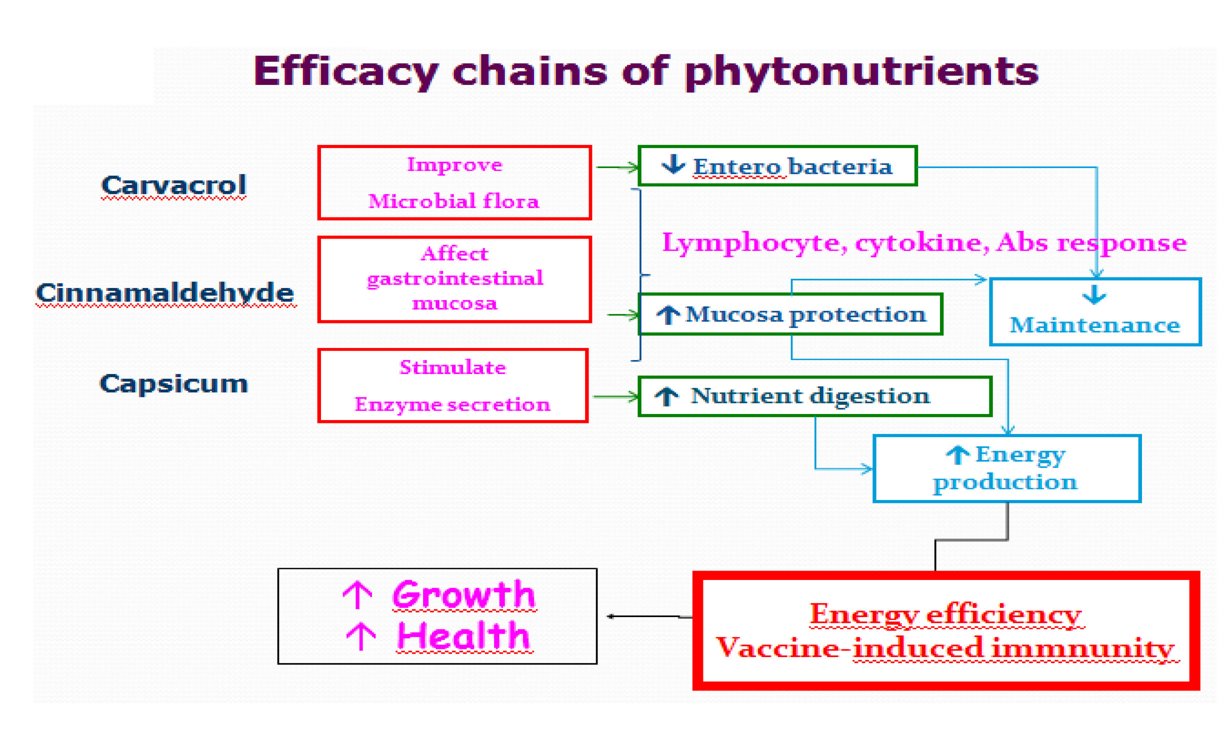


Fig. 6. Effect of dietary supplementation with VAC on intestinal cytokine mRNA levels at DPI 0.



ACKNOWLEDGMENT

This work has been supported by the Trust agreement between Pancosma and ARS. We thank Stacy Torreyson, Ashley Cox, and Margie Nichols for their significant contributions to this research.

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